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Clathrin Coats—Threads Laid Bare

Minireview

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Flux of membrane and proteins through secretory and endocytic pathways in eukaryotic cells is mediated by transport vesicles. Genesis of these vesicles involves assembly of cytoplasmic coat protein complexes onto the donor organelle membrane. Three basic classes of vesicle coats have been identified: clathrin, COPI, and COPII (reviewed in Schmid, 1997, and references therein). Coats are thought to physically deform a patch of the donor organelle membrane into a new vesicle and sequester selected membrane proteins into the emerging vesicle (Figure 1A). Proteins packaged into coated vesicles include cargo in transit to other organelles and machinery to guide and dock each vesicle to the target organelle. Thus, coats have a central role in orchestrating traffic between membrane organelles. Once coated vesicles have formed, coat disassembly allows vesicle fusion with the target membrane and returns coat complexes to the cytoplasm for additional cycles of vesicle formation (Figure 1A). Since their discovery in the 1960s, clathrin coats have served as a paradigm for understanding the molecular basis of vesicle formation (reviewed in Schmid, 1997). Clathrin coats act at the plasma membrane to form endocytic transport vesicles, at the *trans*-Golgi network (TGN) to form endosome-targeted vesicles, and possibly at other transport steps. Recent progress has provided insights into molecular interactions that govern clathrin coat dynamics. Now, ter Haar et al. (1998) report in this issue of *Cell* the crystal structure of the clathrin heavy chain amino-terminal domain, a site of key interactions within clathrin coats.

Clathrin Coat Components

Two oligomeric protein complexes, clathrin and adaptor proteins (APs), are major constituents of clathrin coats (reviewed in Schmid, 1997). Clathrin is a three-legged molecule, formed by C-terminal association of three elongated heavy chains (HC), each carrying a light chain (LC) (Figure 1B). The distinctive clathrin shape, a triskelion, is well-adapted for assembly into polygonal arrays characteristic of clathrin coats (Figure 1C). In low ionic strength, mildly acidic, high Ca^{2+} buffers, purified clathrin triskelia self-assemble into closed polyhedral cages resembling the coats on transport vesicles. Removal of LC subunits from triskelia does not prevent cage formation, indicating that self-assembly is an inherent property of HC (Ungewickell and Ungewickell, 1991, and references therein). However, compared to intact clathrin, LC-free triskelia assemble more readily in the absence of Ca^{2+}. These properties suggest that LC might inhibit spontaneous clathrin assembly, thereby ensuring assembly only at membrane sites destined for vesiculation (Ungewickell and Ungewickell, 1991). LC subunits can be phosphorylated and bind Ca^{2+}, calmodulin, and hsc70, all potential regulatory components (Figure 1B).

AP complexes act in both clathrin assembly and cargo

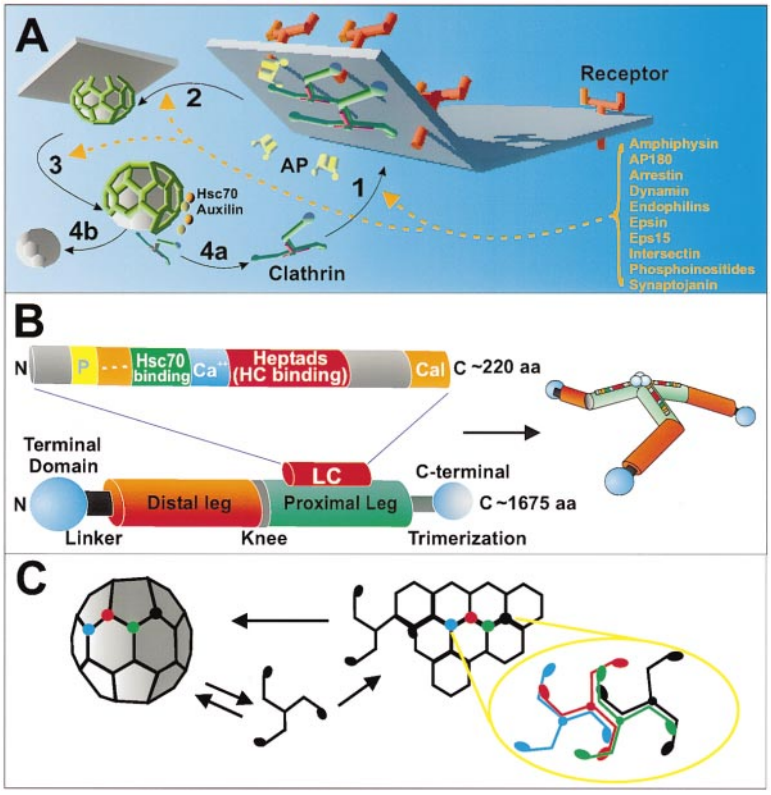

Figure 1. Clathrin Structure and Function

(A) Clathrin-coated vesicle formation. AP complexes associate with the plasma membrane and nucleate assembly of clathrin lattice coats that collect receptor proteins as cargo and drive membrane vesiculation (steps 1–3). Additional factors likely to be involved in coated vesicle formation are shown on the right. Clathrin coat components are recycled by uncoating, probably involving hsc70 and auxillin (step 4a). Uncoated vesicles continue to the target organelle (step 4b).

(B) Domain structure of clathrin HC, LC, and the triskelion. The LC diagram is a composite of domains present in the two forms of mammalian nonneuronal LC. Shown are phosphorylation sites (P), possible regulatory acidic residues (---), heptad repeats (heptads) involved in HC binding, and binding sites for hsc70, calcium (Ca^{++}), and calmodulin (Cal). For illustration, HC C-terminal globular domains are pictured atop the triskelion vertex (Näthke et al., 1992) but may lie underneath as described in Smith et al. (1998).

(C) Clathrin assembly into lattices. Triskelia can assemble into a planar hexagonal array or a closed polyhedron. It is not clear whether vesicles form in vivo by rearrangement of planar lattices or by direct clathrin assembly into a polyhedral cage. Encircled inset depicts the leg arrangements of four triskelia centered at four adjacent vertices.

collection (reviewed in Schmid, 1997). Two related APs have been characterized for clathrin assembly, one localized to the TGN (AP-1) and the other to the plasma membrane (AP-2). The heterotetrameric APs bind to clathrin in vitro and stimulate cage assembly at physiological ionic conditions. Assembly activity is primarily attributed to the AP β subunit, which alone can induce clathrin to form cages. In addition, APs recognize sorting sequences on transmembrane cargo proteins. In this way, APs function as adaptors, linking coat formation to cargo incorporation. In the current view, coated vesicle formation initiates with binding of APs to appropriate membrane sites. In vitro binding studies indicate that AP-1 binding to the TGN is regulated by the GTPase ADP-ribosylation factor 1 (ARF-1) and GTP. In contrast, AP-2 binding to the plasma membrane in vitro is GTP- and ARF-1-independent. A major goal is the identification of membrane components that serve as specific AP-1 and AP-2 binding sites, and characterization of how binding is regulated. Once membrane-associated, APs bind clathrin to nucleate assembly of the polyhedral coat that drives membrane budding (Figure 1A). Phosphorylation and phosphoinositide binding are implicated in regulating aspects of AP function.

Besides APs, specialized adaptors participate in cargo sequestration. Examples include the nonvisual arrestins involved in agonist-induced internalization of the G protein–coupled β-adrenergic receptors (β-AR). Arrestin binds clathrin and β-AR, but does not promote clathrin assembly (Goodman et al., 1997). These characteristics suggest that arrestins act in addition to APs during endocytic coat formation.

Recently, accessory factors that function in endocytic coated vesicle formation have been identified (Figure 1A; reviewed in Cremona and De Camilli, 1997). Generally, these proteins associate transiently with the clathrin coat during vesiculation. Two proteins, dynamin and synaptojanin, have defined enzymatic activities. Dynamin is a GTPase that forms a ring around the neck of a budding coated vesicle. GTP hydrolysis constricts the ring, leading to membrane scission and release of a free vesicle (Figure 1A, step 3; Sweitzer and Hinshaw, 1998). Synaptojanin is an inositol 5-phosphatase, further implicating phosphoinositides in endocytosis. Dynamin and synaptojanin may be targeted to assembling coats by binding to amphiphysin, which also interacts with clathrin and AP-2. Disruption of these interactions by expression of individual amphiphysin domains interferes with endocytosis, providing support for this idea (Slepnev et al., 1998, and references therein). Another example of interactions between accessory factors and structural coat components stems from studies of epsin (Chen et al., 1998). Epsin binds clathrin and AP-2, and was identified as a binding partner for the accessory protein, Eps15. Expression of Eps15- or AP-2-binding domains from epsin inhibits endocytosis. These studies define an elaborate network of accessory proteins linked to clathrin and APs that may be necessary for coat assembly, membrane invagination, and fission (Figure 1A). Phosphorylation may also regulate assembly of this network (Slepnev et al., 1998).

After vesicle formation, the clathrin coat is disassembled and the components recycled to the cytoplasm (Figure 1A, step 4a). Both in vitro and in vivo evidence support a role for the chaperone hsc70 in uncoating (reviewed in Schmid, 1997). Auxilin, a coated vesicle–associated protein, is also required for coat disassembly in vitro. Together with auxillin, hsc70 disassembles LC-free cages in vitro, arguing against proposals that hsc70-mediated uncoating requires binding to LC.

Clathrin Coat Structure

With identification of the major coat components and accessory factors, the challenges now are to define the nature and sequence of interactions leading to coat assembly (and disassembly) and understand how this complex choreography is regulated. We focus here on recent efforts to characterize HC domains responsible for self-assembly and efforts to establish the structural basis for regulation by LC and APs.

By electron microscopy (EM), a clathrin triskelion displays distinct domains (Figure 1B; reviewed in Schmid, 1997). At the base of each leg is the N-terminal globular domain, connected by a linker to the extended distal segment. The distal segment is separated from a similarly extended proximal segment by a bend (knee). HCs trimerize at the vertex and may end with C-terminal globular domains. The vertex and proximal segment constitute the triskelion "hub." Proximal segments splay out from the vertex in a downward direction giving the molecule a tripod appearance.

A new view of in vitro–assembled coats at 21 Å, achieved by cryo-EM, shows individual clathrin molecules and the arrangement of their domains within the lattice (Smith et al., 1998). A single triskelion is centered at each vertex in the coat (Figure 1C). The proximal segment of an individual HC extends to the neighboring vertex, where the bend at the knee directs the distal segment underneath the vertex and along the next edge to the next adjacent vertex. Here, the terminal domain dips inward, forming a hook-like projection that contacts APs. With this arrangement of triskelia, each polyhedral edge is formed by anti-parallel proximal segments from adjacent vertices and antiparallel distal segments from vertices once-removed from the edge (Figure 1C).

Analysis of Clathrin Structure and Assembly Properties

Early studies suggested that sequences involved in trimerization, LC binding, and cage assembly are located within the HC hub region (reviewed in Schmid, 1997). Recently, refined mapping of proteolytic cleavage products, analysis of recombinant HC fragments, and ultrastructural localization of monoclonal antibody epitopes have yielded a more precise definition of functional domains within the hub (Näthke et al., 1992; Liu et al., 1995).

HC sequences specifying trimerization were mapped to residues 1550–1589, close to the C terminus. HC fragments containing this domain form trimers in the absence of LC when fused to maltose-binding protein and expressed at high levels in *E. coli*. Although trimerization can occur without LC, characterization of yeast LC-deficient mutants and HC trimerization mutants suggests that LC contributes to trimer formation or stability at normal expression levels in vivo (Chu et al., 1996; Huang et al., 1997; Pishvaee et al., 1997). It is not clear whether the role for yeast LC in trimerization is unique or is shared by mammalian LC.

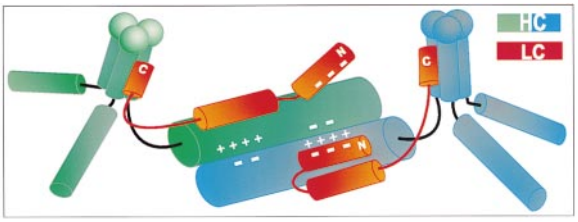

Figure 2. Speculative Models for Regulation of Clathrin Self-Assembly

Please see text for details. Only hubs and one LC per hub are shown. Cylinders represent regions predicted to form α helices. The diagram is schematic and does not take into account the likely possibility of higher-order folding patterns in HC. Acidic (−) and basic (+) residues involved in establishing and regulating ionic interactions are indicated. The LC C terminus is shown associated with the HC trimerization domain where it could potentially regulate trimerization and self-assembly.

Within the proximal domain, an LC-binding region spans ~270 residues adjacent to the trimerization domain (aa 1213–1481). While there is agreement that the LC C terminus lies at the hub vertex, the position of the N terminus is unresolved. One report places the LC N and C termini in proximity near the vertex, based on the observation that two antibodies specific for opposite ends of LC interfered with each other for binding to triskelia (Näthke et al., 1992). However, a subsequent immuno-EM study mapped LC N-terminal epitopes near the knee, not the vertex (Kirchhausen and Toyoda, 1993). The issue may have important implications since the LC N-terminal region contains potential regulatory features (Figure 1B).

Effects of HC mutations on trimerization and LC binding in yeast generally support the domain organization established by in vitro studies of mammalian clathrin (Pishvaee et al., 1997). However, some mutations in the trimerization domain increased or decreased LC binding without strong effects on trimerization. On an α helix model of the trimerization domain, these mutations fall along one face, opposite the face containing trimerization mutations. These results argue that the LC C terminus extends into the trimerization domain, providing a structural basis for the role of LC in trimerization (Figure 2). It was also speculated that, by bridging the trimerization and proximal domains in the hub, LC could influence the conformational flexibility of triskelion legs as they emerge from the vertex. Changes in vertex flexibility, mediated by regulatory inputs to LC, could alter assembly properties of clathrin by affecting alignment of neighboring clathrin legs in the lattice, a model that awaits testing (Pishvaee et al., 1997; Figure 2).

A new hypothesis for regulation of HC self-assembly has emerged from studies of recombinant clathrin hubs (Liu et al., 1995; Ybe et al., 1998). Self-association of purified recombinant hubs into irregular lattice-like structures maps assembly properties to the proximal domain, and indicates an important contribution of HC distal regions in promoting assembly of triskelia into well-ordered arrays. The LC inhibits hub assembly under physiological pH and Ca^{2+} conditions. Mutation of three acidic amino acids in the N-terminal region of LC (aa 23–25) to uncharged residues eliminated inhibition at neutral pH (Ybe et al., 1998). In low ionic strength conditions, hub assembly was shown to involve salt-sensitive interactions between associating HC. These findings prompted a "salt-bridge" hypothesis for HC self-assembly in which cage assembly involves ionic interactions between patches of conserved acidic and basic residues in adjacent HC proximal segments. Regulation of assembly by the acidic LC residues was proposed to occur through reversible neutralization of the HC basic residues (Figure 2). In this scenario, the contradictory placements of the LC N terminus, either toward the knee or toward the vertex, could represent reversible regulatory conformations of LC (Figure 2). While the salt-bridge model is appealingly simple, no structural or functional data yet identify specific HC residues involved in salt-bridge formation, or establish neutralization of HC charged residues by LC. Site-directed mutagenesis and an atomic resolution structure of the hub will be essential to define the molecular contacts contributing to the assembly process.

Structural assembly models, derived from self-assembly studies, must also consider APs, which mediate coat formation in vivo and in vitro under physiological ionic conditions (reviewed in Schmid, 1997; Cremona and De-Camilli, 1997). Little is known about the structural basis for AP stimulation of clathrin cage assembly. APs interact with both the hub and the terminal domain of clathrin. However, APs do not stimulate hub assembly, indicating that AP binding to the terminal domain is required for the assembly process. This binding may be important to promote HC–HC contacts and/or to position APs at the hub to reverse the inhibitory effects of LC on assembly.

Crystal Structure of the Clathrin Terminal Domain

The HC terminal domain is a key nexus between cage assembly and cargo collection. Binding of this domain by APs is required for in vitro cage assembly under physiological ionic conditions. The terminal domain remains in contact with APs in the assembled coat, forming part of the bridge connecting the outer clathrin cage to the membrane (Smith et al., 1998). The cargo-specific arrestin adaptors also contact the coat through the terminal domain (Goodman et al., 1997). Recent NMR analysis revealed that a peptide bearing the LDL receptor endocytosis sorting signal interacts with the terminal domain (Kibbey et al., 1998). This unanticipated result suggests that clathrin, through its terminal domain, may act directly to collect certain membrane cargo molecules.

Kirchhausen and colleagues (ter Haar et al., 1998) have now determined the crystal structure of the HC terminal domain and flanking linker sequences to 2.6 Å resolution, offering an unambiguous structural basis for the multiple binding activities. The terminal domain is a seven-blade β propeller, formed by circular arrangement of seven four-stranded β sheets. The location of the arrestin interaction site illustrates how the structure is suited for adaptor binding (ter Haar et al., 1998). Terminal domain residues required for β-arrestin binding (Goodman et al., 1997) form a patch at the edge of a prominent groove between the first and second blades. The disposition of basic and hydrophobic side chains in this patch

matches the acidic and hydrophobic residues in arrestin required for terminal domain binding (Krupnick et al., 1997). Mutation of arrestin-binding residues in the terminal domain does not prevent AP-2 binding (Goodman et al., 1997), leading ter Haar et al. (1998) to propose that APs might bind to another groove in the propeller. Although apparently binding to distinct sites, arrestin and the AP-2 β subunit contain similar sequences corresponding to a clathrin-binding motif common to several other proteins, including amphiphysin and epsin (Dell'Angelica et al., 1998; Kay et al., 1998). The terminal domain structure should facilitate mutagenesis studies to determine the basis for differential recognition of these similar clathrin-binding sequences in distinct adaptors and accessory proteins.

The crystallized clathrin fragment also contains part of the linker that connects the terminal domain to the distal leg. This linker assumes an α zigzag pattern of ten α helices extending away from one face of the terminal domain propeller at an acute angle. The overall appearance of the crystallized fragment resembles the hook-like extensions of the terminal domain visualized in assembled coats by cryo-EM. Like the terminal domain, the linker segment does not contain sequence repeats that predict a repetitive substructure. This is in contrast to tetratricopeptide and ankyrin repeats that also form helical zigzags (see ter Haar et al., 1998, for references). The authors note that the linear density of amino acids in the linker zigzag is the same as that calculated for the proximal and distal leg segments and suggest that the other portions of the clathrin leg may also consist of a zigzag structure.

Conclusions

The crystal structure of clathrin has been a long-standing goal in the field. Efforts to crystallize the full-length molecule have not been fruitful. Nevertheless, with success in determining the crystal structure of recombinant terminal domain and linker, and production of recombinant hubs, a divide-and-conquer crystallization strategy may ultimately yield a complete view of the clathrin molecule at atomic resolution. Together, a map of triskelion packing in the clathrin cage, and a description of the folding pattern of HC and LC subunits in the triskelion, will provide a structural framework for understanding clathrin assembly properties and regulation by LC and APs. Onto this framework it will be necessary to incorporate the activities of the increasing array of accessory factors that participate in coat assembly, cargo selection, membrane vesiculation, and uncoating. How do each of these proteins associate with nascent coats? At what stage of vesiculation are individual factors recruited and how do they affect coat dynamics? What are the regulatory influences of phosphoinosotides and protein phosphorylation? What other regulatory factors harness the self-assembly properties of clathrin so that coat formation occurs at appropriate membrane sites? What triggers uncoating and how is coat disassembly restricted until vesiculation is complete? Answers to such questions will allow integration of complementary functional and structural data into a better understanding of clathrin-mediated protein transport.

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